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Abstract: Neurodegenerative diseases are frequently associated with hypoxic conditions. During hypoxia the neuronal cytoskeleton is rapidly reorganized and such abnormalities are directly linked to adverse outcomes. Besides their roles as master regulators of the cytoskeleton, the Rho GTPases are also involved in cellular processes stimulated by hypoxic stress. We investigated the contribution of Rac1-mediated signaling to hypoxic responses of mature neurons using primary cortical cells cultured for 17 days in vitro. We show Rac1 is both upregulated and activated during hypoxia. Pharmacological inhibition of Rac1, but not RhoA, completely abrogated hypoxic HIF-1 stabilization and expression of the HIF-1 targets VEGF and GLUT1. Furthermore activity of JNK and GSK3 were also highly dependent on Rac1 activity and biphasic effects were observed after 6 and 24 h of exposure. Notably, inhibition of either pathway suppressed HIF-1 accumulation. Although inhibition of Rac1 did not affect neuronal viability during acute exposure cell death was strongly induced after 24 h revealing a time-dependent effect of Rac1 signaling on survival. Thus hypoxia-activated Rac1 is critical for neuronal HIF-1 stabilization and survival during oxygen deprivation via integration of complex signaling cascades.

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Temporal Rac1 – HIF-1 crosstalk modulates hypoxic survival of aged neurons

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Running Title: Rac1 and HIF-1α crosstalk

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Abstract

Neurodegenerative diseases are frequently associated with hypoxic conditions. During hypoxia the neuronal cytoskeleton is rapidly reorganized and such abnormalities are directly linked to adverse outcomes. Besides their roles as master regulators of the cytoskeleton, the Rho GTPases are also involved in cellular processes stimulated by hypoxic stress. We investigated the contribution of Rac1-mediated signaling to hypoxic responses of mature neurons using primary cortical cells cultured for 17 days *in vitro*. We show Rac1 is both upregulated and activated during hypoxia. Pharmacological inhibition of Rac1, but not RhoA, completely abrogated hypoxic HIF-1α stabilization and expression of the HIF-1 targets VEGF and GLUT1. Furthermore activity of JNK and GSK3β were also highly dependent on Rac1 activity and biphasic effects were observed after 6 and 24h of exposure. Notably, inhibition of either pathway suppressed HIF-1α accumulation. Although inhibition of Rac1 did not affect neuronal viability during acute exposure cell death was strongly induced after 24h revealing a time-dependent effect of Rac1 signaling on survival. Thus hypoxia-activated Rac1 is critical for neuronal HIF-1α stabilization and survival during oxygen deprivation via integration of complex signaling cascades.

Keywords: HIF-1α, JNK, GSK3, neuronal survival, *in vitro*

1. Introduction

Neurons are very sensitive to changes in local oxygen tension due to their high oxygen consumption rates (Noh et al., 2012). Since stroke and many neurodegenerative diseases are also characterized by oxygen deprivation understanding the neuronal response to hypoxia is essential to develop strategies to prevent injury or improve brain function after hypoxic insult. Adaptation to hypoxia on the cellular level is largely regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) (Engelhardt et al., 2014; Iyer et al., 1998). HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1β (also known as aryl hydrocarbon receptor nuclear translocation, ARNT) and an oxygen-regulated HIF-1α subunit (Wang and Semenza, 1995). During normoxic conditions HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylases (PHDs) - a modification that leads to recognition of HIF-1α by the von Hippel-Lindau (VHL) protein and proteasomal degradation (Cockman et al., 2000). When oxygen levels drop the PHDs are inhibited, HIF-1α accumulates in the cytoplasm and translocates into the nucleus where it dimerizes with its β-subunit to form a functional HIF-1 protein (Jaakkola et al., 2001; Kallio et al., 1998). Together with additional co-factors HIF-1 binds to the hypoxia-response element (HRE) of its target genes and initiates their transcription (Jiang et al., 1996). By inducing glycolytic enzymes like phosphofructokinase and angiogenic factors like erythropoietin and vascular endothelial growth factor HIF-1 can confer tolerance to hypoxia (Bernaudin et al., 2002; Engelhardt et al., 2014). However HIF-1 contributes also to hypoxia-induced cell death by inducing pro-apoptotic Bcl-2 family members or by stabilizing the tumor suppressor p53 (Halterman et al., 1999; Kim et al., 2004).

Additional post-translational modifications like sumoylation and phosphorylation also influence HIF-1 stability, localization and transcriptional activity showing that its regulation is a complex process (Berta et al., 2007; Engelhardt et al., 2014; Flugel et al., 2012).

Rho GTPases are key regulators of the cytoskeleton but additionally are involved in various cellular processes including survival and death. Interestingly some family members are regulated by hypoxia in a manner similar to HIF-1α. Rac1, RhoA and Cdc42 were shown to be upregulated after a few hours of oxygen deprivation (Turcotte et al., 2003) and it was demonstrated that different Rho GTPases regulate the stabilization of HIF-1α in different non-brain cell types during hypoxia (Xue et al., 2006; Zhang et al., 2009). Indeed hypoxia-activated Rac1 is crucial for HIF-1 activity in Hep3B cells (Hirota and Semenza, 2001) and in turn HIF-1 binds to the Rac1 promoter and enhances its expression in pulmonary artery smooth muscle cells (Diebold et al., 2010). Particularly in the brain however it is unclear whether a similar interaction between Rac1 and HIF-1 exists and/or via which pathways the proteins might be connected.

This study investigated whether Rac1 modulates HIF-1-driven hypoxic adaptation of mature primary cortical neurons and aimed to identify candidates that mediate the crosstalk between those pathways to gain a better insight into mechanisms of neuronal death during oxygen deprivation.

2. Results

2.1 Rac1 is upregulated and activated by hypoxia

It has been shown in different cancer cell lines like Caki-1, MKN-45 and HepG2 (Turcotte et al., 2003; Xue et al., 2006) that Rac1 is induced by hypoxia. We exposed mature primary cortical mouse neurons to 1% oxygen for short periods of time to investigate if neuronal Rac1 is similarly modulated. Western blot analysis and quantification showed that 4h and 6h of hypoxia increased Rac1 protein levels compared to normoxic control samples (Fig. 1A). Immunocytochemistry confirmed this observation. Normoxic cells showed very weak staining for Rac1 whereas hypoxic cells gave a bright and clear signal (Fig. 1B). Rac1 G-LISA further demonstrated that activation of Rac1 is hypoxia-dependent. Increased Rac1 activity was observed after 2h (142.8 ± 7.2; $p<0.01$) and remained elevated up to 6h (147.9 ± 18.5; $p<0.01$) compared to normoxic controls (Fig. 1C). Thus hypoxia not only elevates neuronal Rac1 protein levels but also increases its activity.

2.2 Rac1 is crucial for HIF-1-driven adaptive hypoxic response

Since Rac1 is strongly induced by oxygen deprivation we pharmacologically investigated its contribution to the neuronal hypoxic response. As expected, Western blot analysis showed stabilized HIF-1α protein after 4h and 6h hypoxic exposure however treatment with a specific Rac1 inhibitor completely abrogated HIF-1α accumulation (Fig. 2A). In contrast a RhoA inhibitor had no major effect on HIF-1α stabilization (Supplementary Fig. 1A). Immunostaining also showed basal nuclear normoxic HIF-1α levels were increased during hypoxic exposure and that Rac1 inhibition strongly suppressed hypoxic nuclear HIF-1α

accumulation (Fig. 2B). To further confirm that Rac1 is required for HIF-1-driven hypoxic adaptation, expression of HIF-1α and its target genes GLUT1 and VEGF was analyzed by quantitative real-time PCR (Fig. 2C&D). Normoxic mRNA expression levels of both proteins, as well as HIF-1α mRNA levels (Supplementary Fig.1B), were unaltered by Rac1 blockade. Notably, inhibitor treatment abrogated hypoxia-induced expression levels of both GLUT1 and VEGF within 4h exposure (3.37 ± 0.36 and 8.71 ± 0.97 fold respectively; $p<0.01$, (Fig. 2C&D). Thus Rac1 is a potent regulator of neuronal HIF-1-driven cellular hypoxic responses. Hypoxic HIF-1α mRNA levels remained unchanged (Supplementary Fig.1B).

2.3 JNK is involved in Rac1-dependent HIF-1α regulation

Rac1 activates JNK signaling cascades (Minden et al., 1995) and in HeLa cells JNK phosphorylation during hypoxia was important for HIF-1 transcriptional activity (Comerford et al., 2004). Since protein levels of prolyl hydroxylase 2 (PHD2) and von Hippel Lindau (VHL), proteins known to be involved in HIF-1 degradation, were also largely unchanged (Supplementary Fig 1C&D), we assessed whether neuronal Rac1-driven HIF-1α stabilization could be mediated by JNK. After 4h of hypoxia phospho/total JNK levels were marginally elevated (120.6 ± 4.6 $p<0.05$). Treatment with 50 or 100 μM Rac1 inhibitor dramatically decreased JNK phosphorylation in normoxia (50.8 ± 6.5; $p<0.01$) and hypoxia (4h: 76.0 ± 7.0, $p<0.05$ and 37.8 ± 17.5, $p<0.0001$; 6h: 72.3 ± 13.5 and 46.7 ± 14.8, $p<0.01$) while total levels remained constant (Fig. 3A). Next, to determine whether JNK is involved in HIF-1α induction, we treated cells with the JNK-specific inhibitor SP600125 prior to hypoxic exposure. Although not totally prevented, hypoxic

accumulation of HIF-1α was significantly reduced after 4h and 6h by approximately 30% ($p<0.05$) compared to untreated controls (Fig. 3B). Immunocytochemistry confirmed these findings wherein hypoxic HIF-1α staining was reduced by JNK blockade in comparison to untreated cells after 6h (Fig. 3C). Taken together neuronal JNK activity is dependent on Rac1 and partially contributes to HIF-1α induction during hypoxia.

2.4 Active GSK3 modulates hypoxic HIF-1α accumulation in neurons

Studies in HepG2 cells (Flugel et al., 2012) suggest another potential link between Rac1 and HIF-1α could be Glycogen synthase kinase 3 (GSK3). We assessed if GSK3β - the isoform highly expressed in CNS neurons - is Rac1 dependent by monitoring phosphorylated (Ser9) and total GSK3β protein levels with and without Rac1 inhibition (Fig. 4A). After 6h of hypoxia GSK3β phospho/total ratio was significantly decreased (73.8 ± 4.1, $p<0.05$) compared to normoxia. In contrast Rac1 inhibition substantially increased GSK3β phosphorylation und thus inactivated the kinase under both normoxic (208.9 ± 68.3) and hypoxic conditions (4h: up to 272.3 ± 84.2 $p<0.01$; 6h: up to 353.6 ± 98.7 $p<0.0001$) compared to untreated controls. Next, we inhibited GSK3 by treating cells with lithium chloride (LiCL, Fig. 4B) or SB216763 (Fig. 4C) prior to hypoxic exposure to investigate whether Rac1-dependent modulation of GSK3β could modulate HIF-1α induction. Both LiCl and SB216763 dose dependently reduced hypoxic HIF-1α accumulation after 4h and 6h and reduced hypoxic HIF-1α nuclear localization in comparison to untreated controls (Fig. 4C&D). Thus Rac1-induced GSK3 activation also contributes to neuronal HIF-1α hypoxic induction.

2.5 Rac1 regulates PI3K/AKT pathway

GSK3 is a direct target of AKT that in turn is regulated by phosphatidylinositol 3-kinase (PI3K) (Cross et al., 1995). Since PI3K is also associated with Rac1 signaling (Lin et al., 2011; Zhu and Nelson, 2013) we measured changes in phosphorylation of both PI3K and AKT after Rac1 inhibition. Interestingly phosphorylation of the p85 regulatory subunit of PI3K, widely used to determine PI3K activity, was largely unaltered by Rac1 blockade or decreased oxygen levels (Supplementary Fig. 2). However phosphorylation of p55, another regulatory subunit of PI3K, was dose-dependently enhanced in cells treated with the Rac1 inhibitor (Fig. 5A) with levels increasing more than 2 fold ($p<0.05$) in normoxia and up to 3 fold ($p<0.05$) in hypoxia compared to controls (Fig 5A). Similarly AKT phosphorylation was strongly induced by Rac1 inhibition in both normoxia and hypoxia (Fig 5B).

2.6 Neurons survive acute hypoxia without a HIF-1-driven adaptive response

In the absence of Rac1, neurons failed to induce pathways important for HIF-1 stabilization and hypoxic adaptation. We directly evaluated whether Rac1 inhibition alters neuronal survival during hypoxia. MTT results show Rac1 blockade in normoxia or up to 6h hypoxia did not significantly affect enzyme activity (Fig. 6A). Similarly LDH levels were also unaltered (data not shown) suggesting that cortical neurons can survive acute hypoxia despite absence of Rac1 and HIF-1 driven adaptive gene expression. Notably, RhoA inhibition had no effect on cell survival at any of the time points studied (Supplementary Fig.

3A&B). In contrast, during more prolonged hypoxia of up to 24h both LDH (Fig. 6B) and MTT (data not shown) assays showed sustained Rac1 blockade significantly increased neuronal death. Concomitantly, and in line with these findings, decreased phosphorylation of both GSK3β and AKT was observed indicating a complete reversal of the effects seen after acute injury (Fig. 6C). Thus in absence of Rac1 & HIF-1 neurons can survive acute but not prolonged hypoxic exposure.

3. Discussion

Neurons are highly sensitive to oxygen deprivation and hypoxic injury causes significant dysfunction. This study demonstrates that Rac1 is crucial in this response modulating HIF-1α stability and downstream signaling in fully differentiated primary cortical neurons. JNK and GSK3 participate to achieve full HIF-1α induction. Surprisingly, we also show that mature neurons can survive acute hypoxia in the absence of Rac1 and HIF-1-mediated adaptation. In contrast long term Rac1 inhibition induces significant cell death.

Rac1 controls hypoxic HIF-1α accumulation in different cancer cell lines (Gorlach et al., 2003; Hirota and Semenza, 2001; Xue et al., 2006). However little is known of the contribution of Rac1 to the hypoxic neuronal response despite being of special interest to combat hypoxia-associated brain injury. Our data shows primary cortical neurons respond to oxygen deprivation with increased Rac1 protein levels and activity after acute exposure (up to 6h). Similarly elevated Rac1 levels were observed during comparable hypoxic exposures in Caki-1 cells (Turcotte et al., 2003) and Rac1 and HIF-1α protein levels correlated in hypoxic

retinal pigment epithelial cells (Zhang et al., 2009) and HEK293 cells (Hirota and Semenza, 2001). Neuronal Rac1 inhibition completely abrogated HIF-1α stabilization and accordingly expression of the HIF-1 target genes GLUT1 and VEGF was strongly suppressed, underlining that neuronal Rac1 is required for full induction of the cellular response to acute hypoxia. Contrary to observations in HEK293 (Hirota and Semenza, 2001), neuronal Rac1 inhibition did not alter HIF-1α mRNA levels.

Rac1 inhibition did not significantly impact canonical regulators of HIF-1α stabilization. Both PHD2 and VHL levels remained largely unchanged after treatment suggesting other mediators and/or signaling pathways must make an important contribution to outcome. Significantly elevated phosphorylation of neuronal JNK after 4h of hypoxia was observed similar to other studies in HepG2 cells (Minet et al., 2001), N1E-115 neuroblastoma cells (Yamauchi et al., 2006) and hepatocytes (Mollen et al., 2007). Inhibition of JNK diminished HIF-1 transcriptional activity in HeLa cells and reduced HIF-1α protein levels in human bronchial epithelia cells (Comerford et al., 2004). In neurons equivalent outcomes were observed wherein JNK phosphorylation was strongly decreased by Rac1 inhibition and JNK inhibition *per se* reduced HIF-1α levels. Thus in mature neurons hypoxia-induced JNK activation is a Rac1-driven process that modulates HIF-1α stabilization. Although JNK was suggested to be a negative factor for HIF-1α accumulation in primary rat neurons (Antoniou et al., 2010), most studies indicate a positive correlation between active JNK and HIF-1 signaling similar to our findings. Differences in experimental design and cells may underlie this inconsistency.

We identified GSK3β as another mediator of Rac1-driven HIF-1α stabilization. GSK3β is particularly abundant in the CNS (Jin et al., 2006) and in renal carcinoma cells directly phosphorylated HIF-1α thereby targeting it for ubiquitinylation and subsequent proteasomal degradation (Flugel et al., 2007). Furthermore, GSK3 is a direct target of the PI3K/AKT pathway and involved in HIF-1 regulation during hypoxia (Zhong et al., 2000), and tightly associated with Rac1 signaling in COS 7 and natural killer cells (Jiang et al., 2001; Murga et al., 2002). Neuronal Rac1 blockade strongly increased GSK3β serine phosphorylation and abrogated HIF-1α accumulation whereas direct inhibition of GSK3 suppressed HIF-1α stabilization. Hence in neurons hypoxia, Rac1 activation, HIF-1α induction and GSK3 activation are positively correlated. Interestingly, in non-neuronal cells, increased serine phosphorylation and thus inactivation of GSK3 has been correlated with enhanced HIF-1α levels (Flugel et al., 2012; Schnitzer et al., 2005). Thus we demonstrate for the first time that active GSK3 can contribute to acute hypoxic HIF-1α stabilization. Future studies need to explore the molecular mechanisms of this likely cell type specific signaling event.

A major regulatory pathway of GSK3 activity is PI3K/AKT (Cross et al., 1995) that both influences Rac1 activity (Zhu and Nelson, 2013) and is a downstream target of Rac1 (Lin et al., 2011). Although hypoxia had no effect, Rac1 inhibition increased phosphorylation of the PI3K p55 subunit, an isoform of PI3K p85 enriched in rodent brain that regulates PI3K activity similar to p85 (Inukai et al., 1996; Pons et al., 1995). Notably, this occurrence correlated with AKT phosphorylation after Rac1 blockade although in contrast hypoxia inactivated AKT. Therefore GSK3β inactivation could underlie activation of the PI3K/AKT

pathway. Since PI3K and AKT activity support neuroprotection (Zhang et al., 2010) such crosstalk would confer acute hypoxic tolerance and likely compensate the absence of HIF-1α signaling at least in the short term.

In general HIF-1 is believed to be protective during acute and mild hypoxia via expression of its target genes like VEGF and EPO (Engelhardt et al., 2014). The fact that we did not observe any alterations in neuronal survival during 6h of hypoxia might be surprising at first glance, but several studies demonstrate that even sensitive cells like neurons do not undergo cell death when oxygen levels are above 0.5 %. For example differentiated RN46A neuronal cells survived exposure to 1% oxygen for 48h (Zhang et al., 2003) and anoxic cell death was induced in primary cortical rat neurons only when glucose concentrations were decreased (Wohnsland et al., 2010). We did observe however that prolonged hypoxia significantly impaired survival of mature neurons and the absence of Rac1 and HIF-1α aggravated cell death. Thus the ability of mature neurons to survive hypoxia is not only time related but increasingly dependent on Rac1/HIF-1 pathway activation. Interestingly, during prolonged hypoxic exposure Rac1 blockade reversed the phosphorylation of both AKT and GSK3 – AKT being inactivated and GSK3 being activated. Clearly the integration of Rac1 signaling on adaptative outcome is highly complex and time-dependent.

Importantly, this study also highlights that the effects of Rac1 inhibition on JNK, PI3K/AKT and GSK3β activity also occur during normoxia and therefore represent a general mechanism of Rac1 action. As such we can infer that basal Rac1 activity is essential for neuronal survival. However it is also clear that hypoxia-induced Rac1 activity, and activation of JNK and GSK3, is also required for neuronal HIF-1α stabilization. Thus additional oxygen deprivation and

regulation of HIF-1 signaling only adds an extra degree of complexity to both the signaling pathway and outcome.

In conclusion Rac1 plays a pivotal role in the neuronal hypoxic response being crucial for HIF-1α accumulation via activation of JNK and GSK3 pathways. Loss of Rac1 and HIF-1 during acute hypoxia is compensated by strong activation of PI3K/AKT but such effects are reversed during more prolonged exposure. The mechanisms by which this switching occurs is now under investigation. Overall these findings give important fundamental insights into neuronal hypoxic adaptation and underline that time-dependent alterations in signaling pathways have strong influences on the outcome of injury. More emphasis and gain of knowledge on such nuances will definitely be required to combat neuronal damage.

4. Experimental procedure

All experiments were performed in accordance with Swiss animal protection laws and University of Zürich institutional guidelines for animal experimentation.

4.1 Reagents and Antibodies

If not stated differently chemicals and reagents were purchased from Sigma-Aldrich (Missouri, USA). All cell culture reagents were purchased from Life Technologies (Zug, Switzerland). The following antibodies were used: Anti HIF-1α (NOVUS, Littleton, USA), anti Rac1, anti β-Actin (Sigma), anti TUJ-1 (COVANCE, Berkeley, CA, USA), anti phospho-GSK3β and anti total GSK3α/β (Millipore, Billerica, MA, USA), anti phospho-JNK and anti total JNK (R&D

Systems, Minneapolis, MN, USA), anti phospho-p55 and p85 PI3K and total p85 PI3K, anti phospho-AKT and anti total AKT (Cell signaling, Danvers, MA, USA).

4.2 Neuronal culture

Primary neuronal cells were obtained from cerebral cortex of C57BL/6 mouse embryos at gestational stage E14 as described previously (Ogunshola et al., 2002). Neurobasal medium was complemented with B27 supplement (1X), AlbuMAX I (0.25g/ml), streptavidin-penicillin (1%), Sodium-Pyruvate 100U/ml and L-Glutamine (0.5 mM). Cells were seeded on poly-L-lysine coated plastic petridishes or glass coverslips in 24 well plates (60,000 cells/cm^2). Neurons were maintained at normal atmosphere (21% O_2, 5% CO_2) in a humidified incubator at 37 °C for 17 days. Half the media was replaced every 6 days.

4.3 Hypoxia experiments and inhibitor treatments

For hypoxic exposure cells were put in a purpose-built hypoxic glove-box chamber (in vivo 400, RUSKINN Technologies, Guiseley, UK) for different time intervals (37°C, 5% CO_2). The oxygen level was maintained at 1%. Inhibitors of Rac1 (Millipore, Billerica, MA, USA) and RhoA (cytoskeleton, Denver, CO, USA) were added just prior to hypoxic exposure. Inhibitors of GSK3β (SB216763, Cayman chemicals, Ann Arbor, MI, USA; LiCl, Sigma) were added 24h before and JNK inhibitor SP600123 was added 1h before cells were subjected to hypoxia. Control cells were incubated under normal conditions (37°C, 21% O_2, 5% CO_2).

4.4. Western Blot

Neurons were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% NP-40) for 10 min and centrifuged at 16 000 rcf for 10 min at 4°C. The supernatant was stored at -20°C. Protein concentration was measured using Bio-Rad assay according to manufacturer`s instructions. 40-60 µg of protein were loaded on SDS-polyacrylamide gels then transferred to nitrocellulose membrane. After blocking in 0.1% TBS-Tween with 5% milk powder or 5 % BSA membranes were incubated overnight at 4°C with primary antibody. Membranes were washed then incubated with an HRP-conjugated secondary antibody for 1h at room temperature. All blots were normalized to TUJ-1 or β-Actin and quantified using ImageJ software.

4.5 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, AG, Switzerland) according to the manufacturer`s protocol. Subsequent cDNA synthesis used ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) then mouse-specific Taq-man-based gene expression assays (Applied Biosystems, Foster City, CA, USA) were utilized to perform real-time PCR (HIF-1α [Mm00468869_m1]; VEGF [Mm00437304_m1]; Glut1 [Mm00441473_m1]; GAPDH [Mm99999915_g1]. All reactions were run in duplicate using ABI 7500 RT-PCR thermocycler. Data were analyzed using the ΔΔCt-Method.

4.6 Immunocytochemistry

Cells were fixed for 5 min in 4% paraformaldehyde in PBS, permeabilized 3 to 5 minutes with 0.1% Triton X-100 then blocked in 10% normal goat serum. Antibodies were applied overnight at 4°C followed by incubation with

fluorescent secondary antibody (AlexaFluor488, AlexaFluor594, Molecular Probes, Leiden, Netherlands) for 1h at room temperature. Cells were counterstained with 10 μM DAPI for 5 min. For F-actin staining cells were incubated in Phalloidin-TRITC for 40 min at room temperature after fixation. Coverslips were mounted in fluorescent mounting medium (Dako, Glostrup, Denmark) then viewed with an Axiovert fluorescent microscope (Zeiss, Germany).

4.7 Rac1 activity assay (G-LISA)

The activation of Rac1 was assessed using the colorimetric G-LISA Activation Assay Biochem Kit (BK125, Cytoskeleton, Denver, CO, USA). All steps were performed according to the manufacturers protocol.

4.8 Cell death assays:

4.8.1 LDH assay: For quantification of cell death the release of lactate dehydrogenase from cells into the culture medium was detected using Cytotoxicity Detection Kit (Roche, Penzberg, Germany) according to the manufacturer`s instructions.

4.8.2 MTT assay: Thiazolyl blue tetrazolium bromide (Sigma) was dissolved in PBS (0.5 mg/ml) and added to cells in a 96 well format. After 1h incubation at 37°C media was replaced with 100 μL DMSO. Absorption was mearured at 595 nm with background correction at 670 nm.

4.9 Statistical analysis

All graphs were created from at least 3 independent experiments and analyzed using GraphPad Prism software. All error bars represent the mean ± standard deviation. For comparison between different time points within a group one-way ANOVA was used to assess statistical significance with Tukey post-hoc test. For comparisons between different groups we performed two-way ANOVA followed by Bonferroni post-hoc test. A value of $p<0.05$ was considered significant.

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Figure legends

Figure 1. Rac1 is upregulated and activated during hypoxia.

Cells were incubated for 4h and 6h under normoxic (Nx) or hypoxic (Hx) conditions. A) Western blot and quantification was used to assess total Rac1 protein levels, TUJ-1 was used as loading control. B) Cellular Rac1 levels were additionally analyzed by immunocytochemistry; green: Rac1, blue: DAPI, scale bar: 50 µm. C) Rac1 activity was determined by G-LISA. ** $p<0.01$ versus normoxia. Data were analyzed using one-way ANOVA followed by Tukey post-hoc test (A).

Figure 2: Rac1 is crucial for neuronal HIF-1α-mediated signaling.

Cells were treated with 50 or 100 µM Rac1 inhibitor just prior to normoxic (Nx) or hypoxic (Hx) exposure. A) HIF-1α protein levels were determined by Western blot, TUJ-1 was used as loading control. B) Normoxic and hypoxic cells with or without 100 µM Rac1 inhibitor were fixed and immunolabeled for HIF-1α (green) and counterstained with DAPI (blue). Scale bar: 50 µm. TaqMan-based RT-PCR was used to analyze expression of the HIF-1α target genes VEGF (C) and GLUT1 (B) after oxygen and/or inhibitor exposure. Data were normalized to GAPDH and compared to normoxic control sample. ** $p<0.01$ versus normoxic control. # $p<0.05$, #### $p<0.0001$ comparison within the group. Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

Figure 3: JNK contributes to Rac1-dependent HIF-1α regulation.

A) The effect of Rac1 inhibition (50µM or 100 µM) under normoxic (Nx) or hypoxic conditions (Hx) on JNK activity was assessed by Western blot. Results

display the ratio of phosphorylated to total protein levels normalized to the loading control TUJ-1 and compared to the normoxic control sample. B) Neurons were pretreated with 50 μM of JNK inhibitor SP600125 for 1h prior to hypoxic or normoxic exposure and HIF-1α levels were compared to untreated samples by Western blot. The graph shows HIF-1α protein levels normalized to the loading control β-Actin and the 4h hypoxic samples were set to 100%. * $p<0.05$ versus Nx control; #$p<0.05$, ##$p<0.01$, ####$p<0.0001$ comparison within the groups. Data were analyzed using one or two-way ANOVA followed by Tukey or Bonferroni post-hoc test. C) Immunocytochemistry assessed cellular HIF-1α levels in normoxia and hypoxia in the presence or absence of 50 μM JNK inhibitor SP600125. Green: HIF-1α, blue: DAPI, Scale bar: 50 μM.

Figure 4: Active GSK3β is critical for neuronal HIF-1α accumulation.

Cells were treated with 50 or 100 μM Rac1 inhibitor und exposed to 4h and 6h of hypoxia (Hx) or kept under normoxia (Nx). A) Western blots were performed to detect phosphorylated and total protein levels of GSK3β. TUJ-1 was used as loading control. GSK3 was inhibited by treatment with 5 mM and 10 mM LiCl (B) or 5 μM and 10 μM SB216763 (C) 24h prior to hypoxic exposure and HIF-1α levels were analyzed by Western blot and quantification. The 4h hypoxic control sample was set to 100% and β-Actin was used as loading control. Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test. * $p<0.05$ versus Nx control. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and #### $p< 0.0001$, comparison within each group. D) Immunocytochemistry shows cellular HIF-1α levels (green) in normoxia and hypoxia in the presence or absence of 10 mM LiCl. Blue: DAPI, Scale bar: 50 μM.

Figure 5: Rac1 may regulate GSK3β activity via PI3K/AKT pathway

Lysates of normoxic or hypoxic cells treated with or without Rac1 inhibitor (50 and 100 μM) were used in Western blot to detect phosphorylated and total protein levels of PI3K p55 (A) and AKT (B). Results are expressed as the ratio of phosphorylated to total protein and data is normalized to TUJ-1. # $p<0.05$, ### $p<0.001$, #### $p< 0.0001$, comparison within the group. Data analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test.

Figure 6: Neurons survive acute hypoxia without Rac1 and HIF-1α.

Cells were treated with Rac1 inhibitor and kept under normoxic (Nx) or hypoxic conditions (Hx). Untreated neurons were used as control. A) MTT assay was used to assess the effect of acute hypoxia (6h) and inhibition of Rac1 on cell death. Data analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test. B, LDH release into the culture medium was used to detect cytotoxicity after exposure to prolonged hypoxia (24h). ** $p<0.01$, *** $p< 0.001$ compared to Nx control. ## $p<0.01$, comparison within the group using one-way ANOVA followed by Tukey post-hoc test. C) Western blot of cell lysates was used to assess HIF-1α levels and changes in phosphorylation of GSK3 and AKT after 24h hypoxia in presence or absence of Rac1 inhibitor.

Supplementary Figure 1: Effect of Rac1 inhibition on HIF-1α mRNA levels and regulators of HIF-1α stabilization.

Cells were exposed to normoxic (Nx) or hypoxic (Hx) conditions with or without Rac1 inhibitor treatment. A) RhoA inhibition (100μM) does not alter HIF-1α

stabilization. B) mRNA levels of HIF-1α were measured using TaqMan based RT-PCR. Protein levels of PHD2 (C) and VHL (D) before and after Rac1 inhibition were assessed by Western blot. Data were normalized to GAPDH and compared to normoxic controls. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test.

Supplementary Figure 2: Effect of Rac1 inhibition on PI3K p85 subunit.

Phosphorylated and total levels of PI3K p85 were assessed by Western blot. The quantitative graph shows the ratio of phosphorylated to total p85 subunit compared to untreated normoxic control. TUJ-1 was used as loading control. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test.

Supplementary Figure 3: RhoA inhibition does not impact neuronal survival.

MTT assay was used to measure cell survival after acute (A) and prolonged (B) RhoA inhibition. ** $p<0.01$, compared to Nx control. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test.

Figure 1

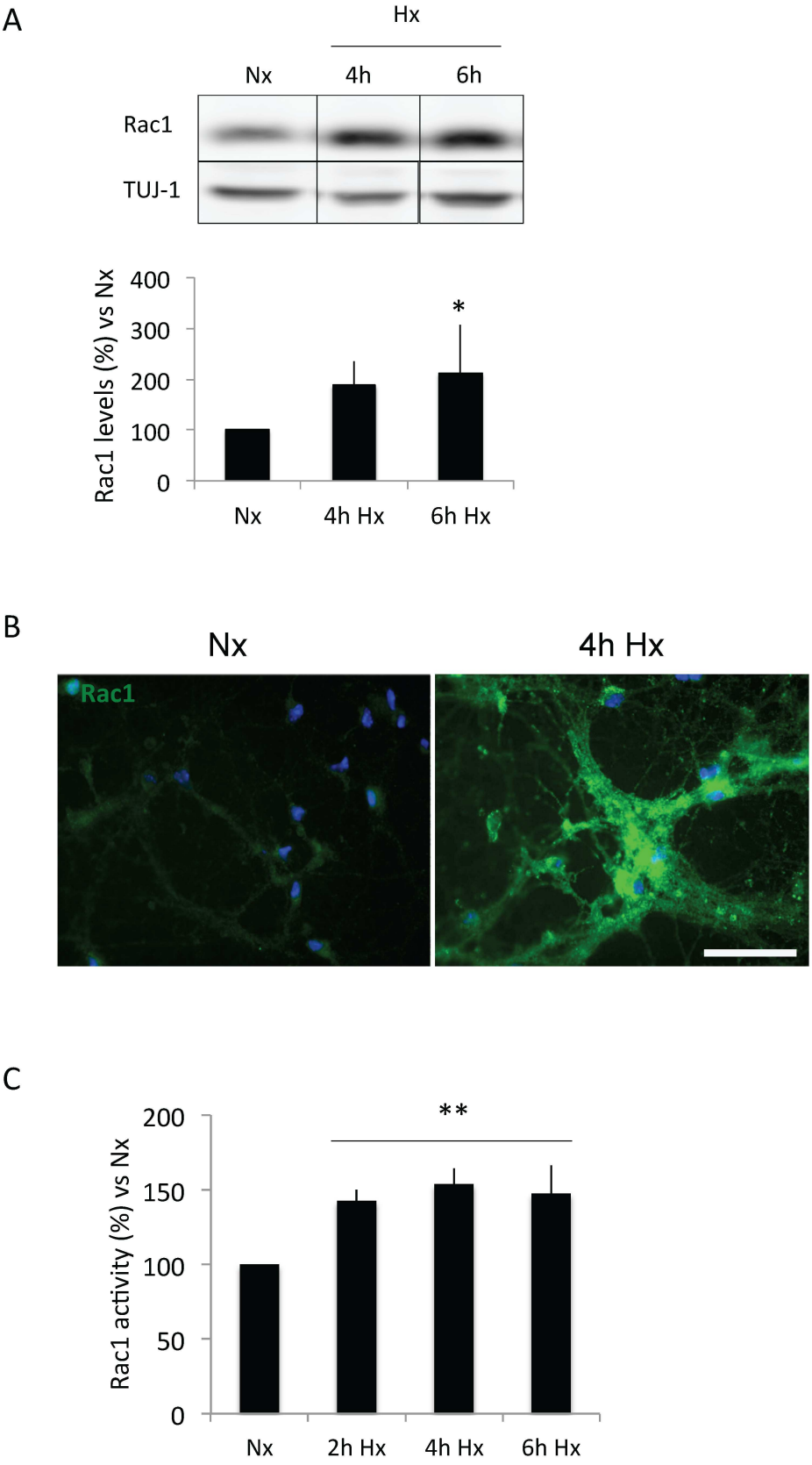
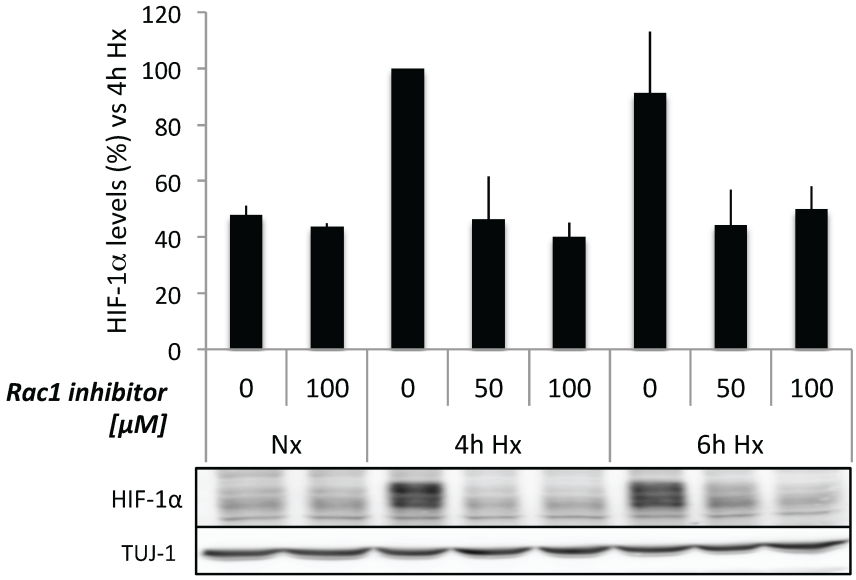

A
Hx
Nx
4h
6h
Rac1
TUJ-1
Rac1 levels (%) vs Nx
400
300
200
100
0
*
Nx
4h Hx
6h Hx
B
Nx
4h Hx
Rac1
C
Rac1 activity (%) vs Nx
200
150
100
50
0
**
Nx
2h Hx
4h Hx
6h Hx

Figure 2

A


HIF-1α levels (%) vs 4h Hx
120
100
80
60
40
20
0
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx
HIF-1α
TUJ-1

B

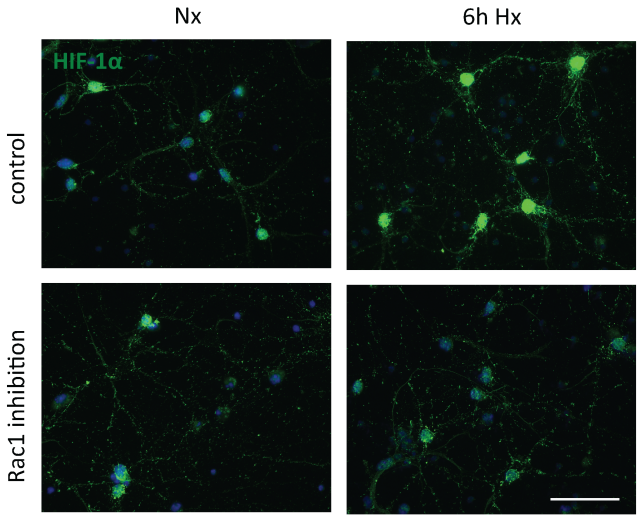
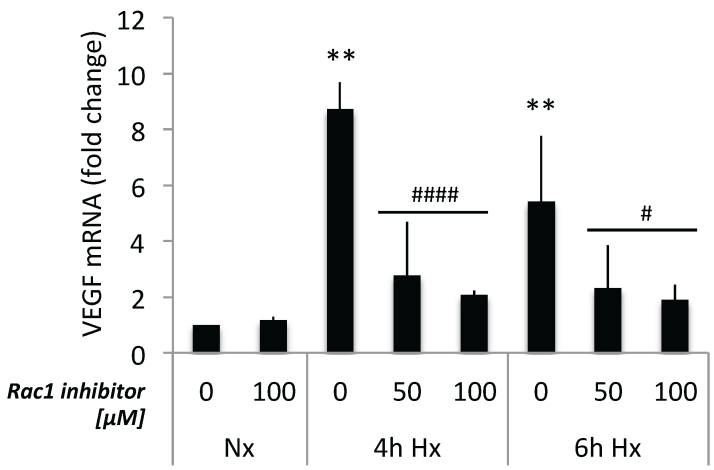

Nx
6h Hx
HIF-1α
control
Rac1 inhibition

Figure 2

C


VEGF mRNA (fold change)
12
10
8
6
4
2
0
**
**
####
#
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx

D

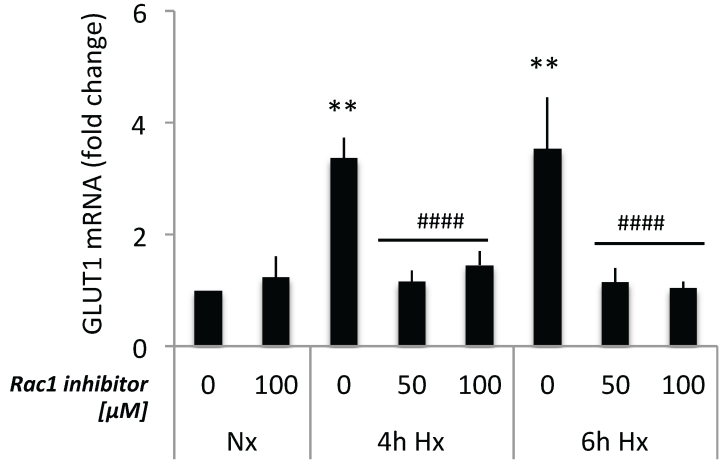

GLUT1 mRNA (fold change)
6
4
2
0
**
**
####
####
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx

Figure 3

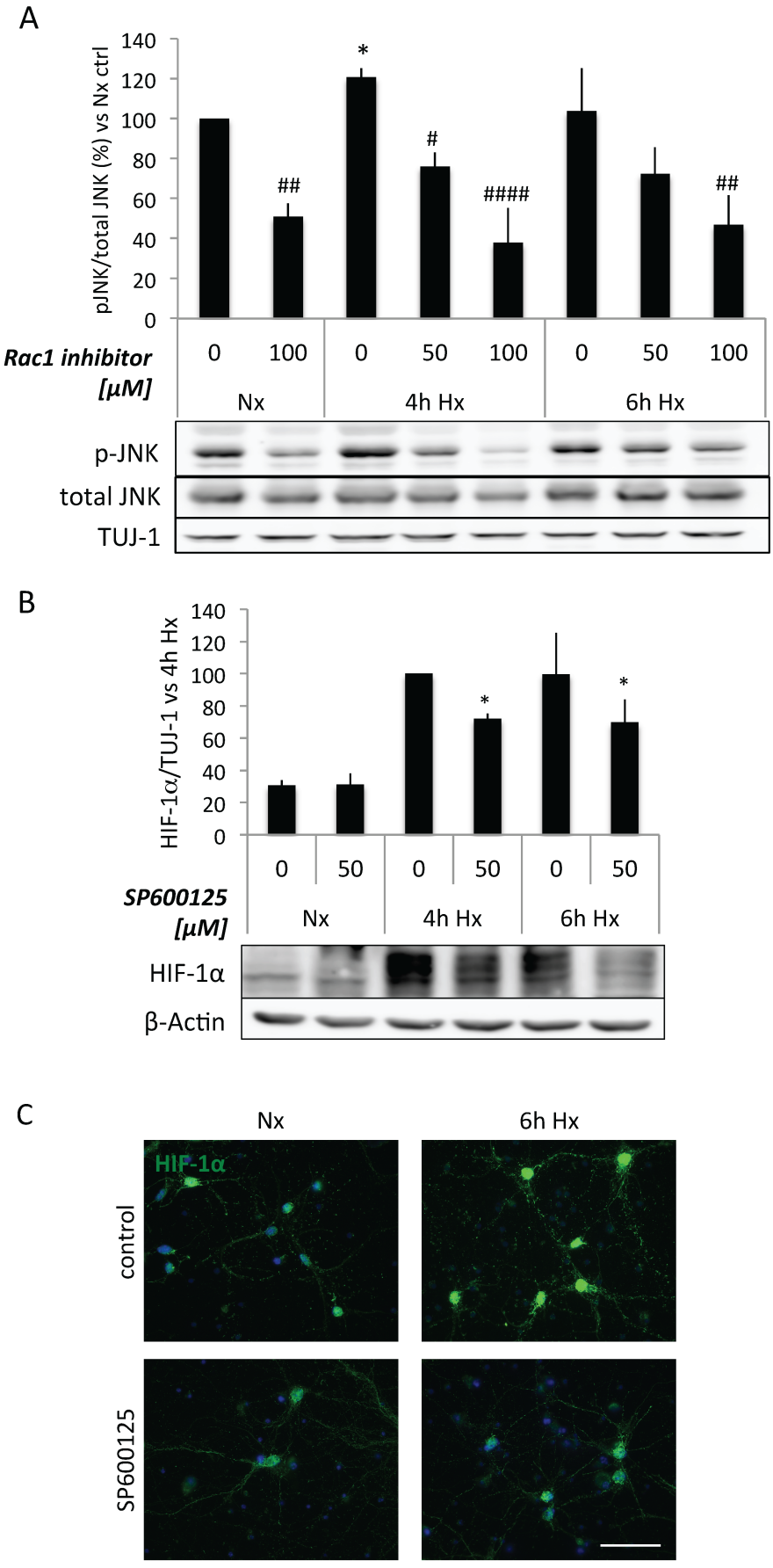

A
pJNK/total JNK (%) vs Nx ctrl
140
120
100
80
60
40
20
0
*
#
##
####
##
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx
p-JNK
total JNK
TUJ-1
B
HIF-1α/TUJ-1 vs 4h Hx
140
120
100
80
60
40
20
0
*
*
SP600125 [μM]
0
50
0
50
0
50
Nx
4h Hx
6h Hx
HIF-1α
β-Actin
C
Nx
6h Hx
HIF-1α
control
SP600125

Figure 4

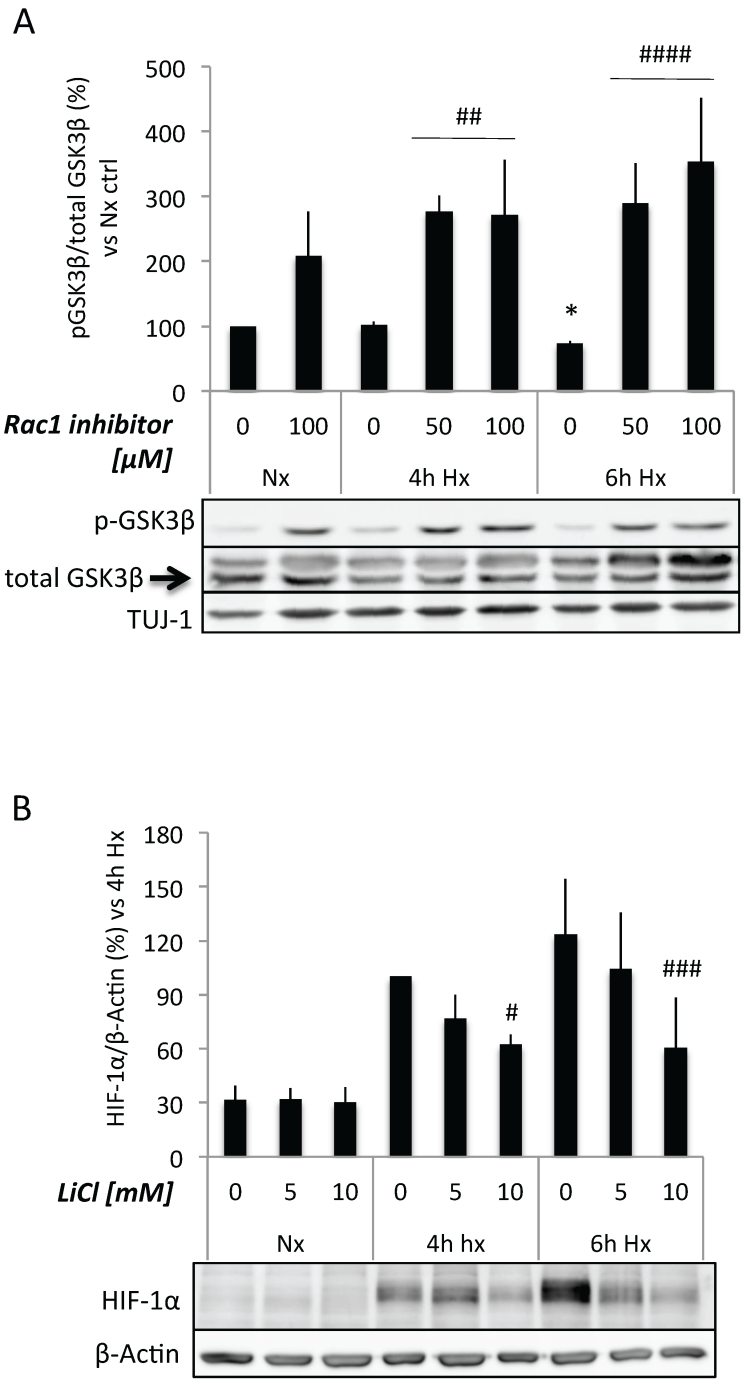
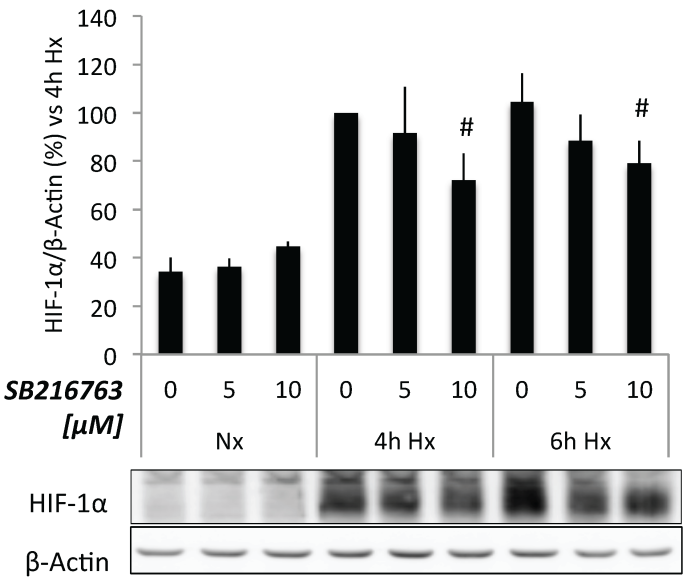

A
pGSK3β/total GSK3β (%) vs Nx ctrl
500
400
300
200
100
0
##
####
*
Rac1 inhibitor [μM]
0 100 0 50 100 0 50 100
Nx
4h Hx
6h Hx
p-GSK3β
total GSK3β
TUJ-1
B
HIF-1α/β-Actin (%) vs 4h Hx
180
150
120
90
60
30
0
#
###
LiCl [mM]
0 5 10 0 5 10 0 5 10
Nx
4h hx
6h Hx
HIF-1α
β-Actin

Figure 4

C


HIF-1α/β-Actin (%) vs 4h Hx
140
120
100
80
60
40
20
0
#
#
SB216763 [μM]
0 5 10 0 5 10 0 5 10
Nx
4h Hx
6h Hx
HIF-1α
β-Actin

D

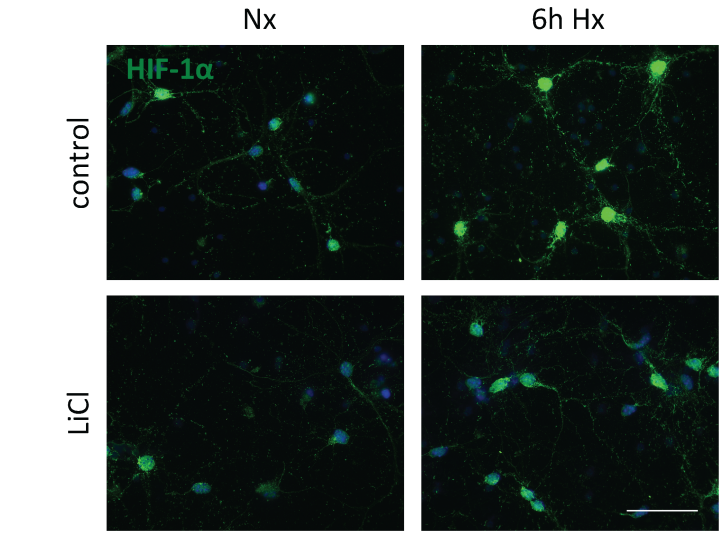
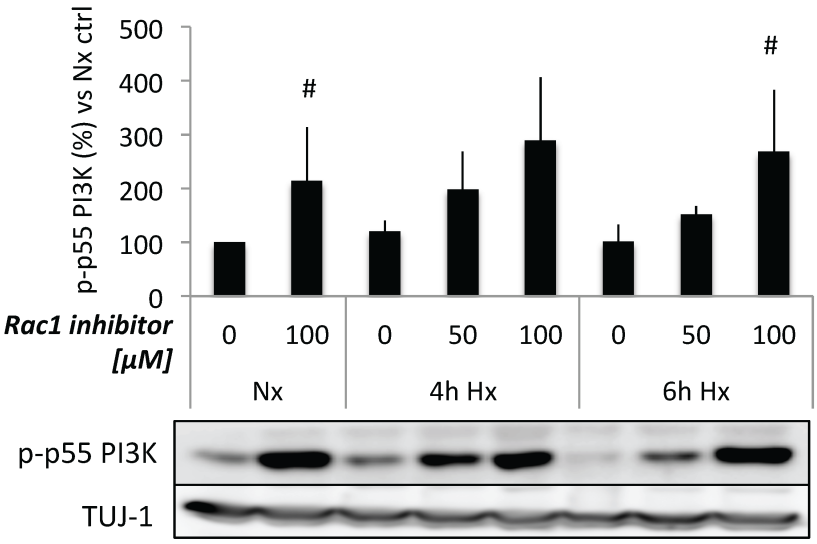

Nx
6h Hx
HIF-1α
control
LiCl

Figure 5

A


p-p55 PI3K (%) vs Nx ctrl
500
400
300
200
100
0
#
#
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx
p-p55 PI3K
TUJ-1

B

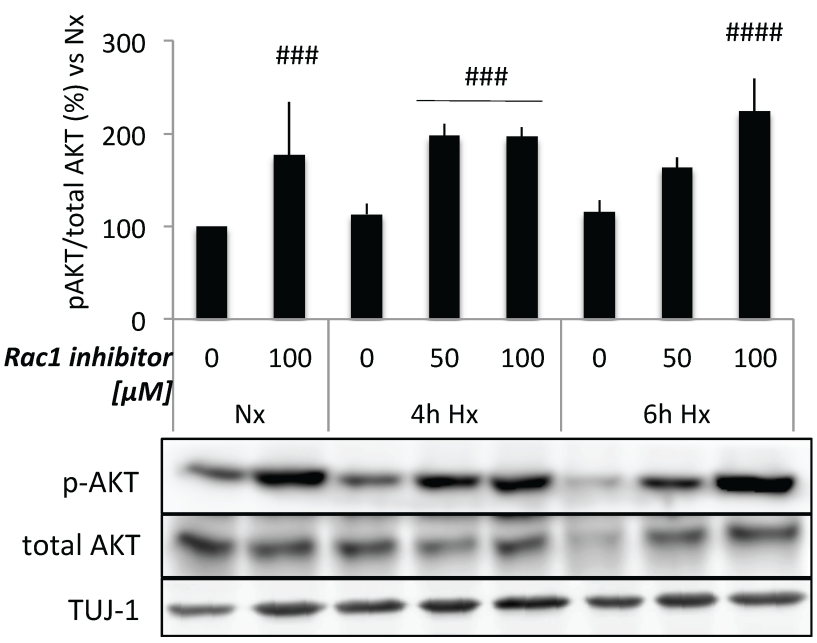
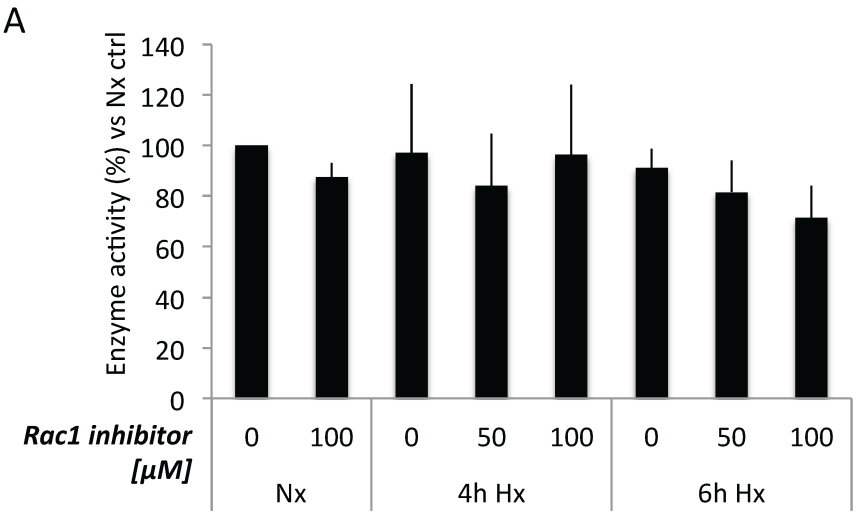
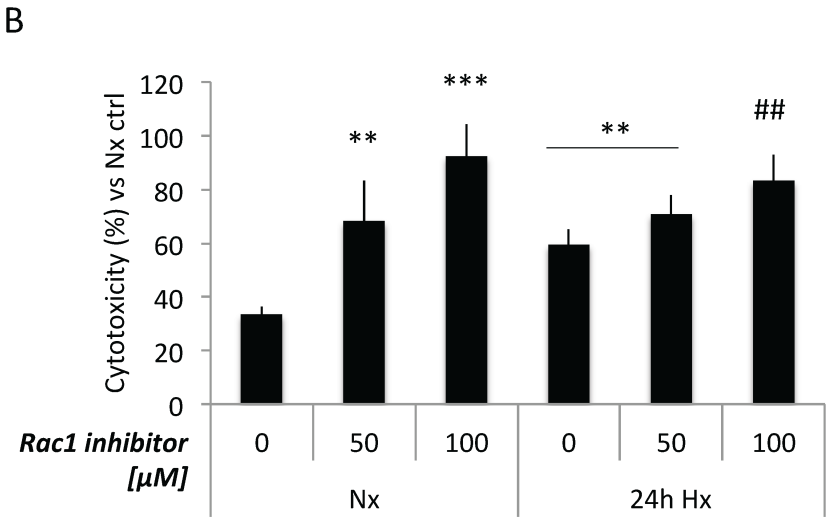
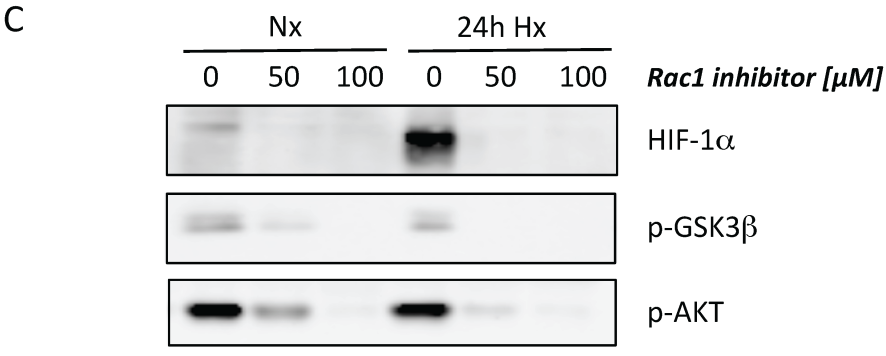

pAKT/total AKT (%) vs Nx
300
200
100
0
###
###
####
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx
p-AKT
total AKT
TUJ-1

Figure 6


A
Enzyme activity (%) vs Nx ctrl
140
120
100
80
60
40
20
0
Rac1 inhibitor [μM]
0
100
0
50
100
0
50
100
Nx
4h Hx
6h Hx


B
Cytotoxicity (%) vs Nx ctrl
120
100
80
60
40
20
0
**
***
**
##
Rac1 inhibitor [μM]
0
50
100
0
50
100
Nx
24h Hx


C
Nx
24h Hx
0
50
100
0
50
100
Rac1 inhibitor [μM]
HIF-1α
p-GSK3β
p-AKT